Purification of Horse-Radish Peroxidase and Comparison of its Properties with those of Catalase and Methaemoglobin

By D. KEILIN and E. F. HARTREE
Molteno Institute, University of Cambridge

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Although the peroxidatic properties of animal and vegetable extracts were recognized already by Schönbein (1863) and ascribed by Bourquelot (1898) to special indirect oxidizing enzymes which Linossier (1898) named peroxidases, the first successful isolation and purification of an enzyme in this category was achieved between 1917 and 1924 by Willstätter and his co-workers. They described the main properties of horse-radish peroxidase and greatly improved the method of estimation of enzyme activity, expressed in terms of 'Purpurogallinzahl', P.Z. or purpurogallin number, P.N. (for a definition see p. 89). The unusually high values of P.N. recorded by Willstätter & Pollinger (1923) can only be ascribed to some error in their calculations. Thus if the total iron (0·064 %) found in their preparation with P.N. = 3070 belongs to the prosthetic group of the enzyme, then, in the light of our present knowledge, it can be estimated that the purity of their most active preparation was probably 40–50 % and that the P.N. values given in their paper are too large by a factor of about 5. However, to obtain at that time a 40 % pure enzyme was in itself a remarkable achievement. It not only paved the way for further study of peroxidase, but stimulated similar work on other enzymes at a time when it was still believed (Warburg, 1928) that owing to their inherent fragility all attempts to isolate and purify intracellular enzymes would have no chance of success. Willstätter's classical study of peroxidase was followed by extensive work on the purification of this enzyme with a view to determining its chemical nature.

The presence of haematin in horse-radish root itself (Keilin, 1927) and in enzyme preparations of widely different purities (Kuhn, Hand & Florkin, 1931; Elliott & Keilin, 1934; Sumner & Howell, 1936) suggested the possibility that the enzyme is a haematin compound. However, it was only after further knowledge had been gained of the properties of methaemoglobin and especially of the compounds it forms with hydrogen sulphide (Keilin, 1933), with sodium azide (Keilin, 1936) and above all with peroxides (Keilin & Hartree, 1935) that the haematin nature of peroxidase was definitely established by Keilin & Mann (1937). This opened the way for a much more precise study of the properties of the enzyme and of the kinetics of its reactions.

Peroxidase offers one of the most instructive examples for the study of the chemical structure of an enzyme. Thus the criteria upon which the haematin-protein nature of peroxidase as well as its close relationship to catalase and to methaemoglobin were established (Keilin & Mann, 1937) can be summarized as follows: (1) Presence in all peroxidase preparations, from horse-radish root itself to the most highly purified enzyme, of a pigment with an acid methaemoglobin-like absorption spectrum. (2) Proportionality between the intensities of the absorption spectra of this methaemoglobin-like pigment or of its pyridine-haemochromogen derivative and the activities (P.N.) of different enzyme preparations. (3) Formation of spectroscopically well defined compounds between this pigment and inhibitors of peroxidatic activity such as potassium cyanide, hydrogen sulphide, sodium fluoride and nitric oxide. (4) Formation of well defined compounds between this haematin pigment and hydrogen peroxide which is the substrate of the enzyme. (5) Rapid decomposition of haematin pigment-peroxide complex and the liberation of the pigment on addition of 'acceptors' like pyrogallol, quinol, ascorbic acid and others which can undergo peroxidatic oxidation. (6) Demonstration of the above reactions between the enzyme and inhibitors or substrate (i.e. hydrogen peroxide) not only with purified enzyme but also with crude root extracts and even slices of horse-radish root.

The peroxidase-peroxide compound provided the first example of direct visual demonstration of union between an enzyme and its substrate. According to the amount of hydrogen peroxide added two distinct red compounds are formed with somewhat different absorption spectra. On the other hand, there is no spectroscopically detectable reaction between peroxidase and substances such as pyrogallol, quinol, etc. Peroxidase also resembles methaemoglobin in changing into a redder alkaline form at higher pH (> 10) and in being reduced by sodium dithionite ($Na_2S_2O_4$) to a ferrous form which can combine with carbon monoxide.

The purest preparation of Keilin & Mann (1937) had P.N. about 950 and contained 1·2 % haemin. It was also shown that milk peroxidase is also a methaemoglobin-like compound which will combine with potassium cyanide, sodium fluoride and hydrogen

peroxide and, after reduction ($Na_2S_2O_4$), with carbon monoxide.

A large-scale preparation of horse-radish peroxidase described by Theorell (1942*a*) enabled him to isolate the enzyme as a pure crystalline material containing 13·2% nitrogen and 1·48% haemin, estimated as pyridine haemochromogen. The molecular weight of the enzyme calculated from the sedimentation and diffusion constants was found to be 44,100 (Theorell, 1942*b*) while the catalytic activity or P.N., estimated in the usual way, was about 930, the highest value reached being 1100. With this preparation Theorell corroborated the main results of the previous work (Keilin & Mann, 1937), especially the haematin nature of peroxidase and the compounds it forms both with its inhibitors and with peroxide. However, in addition to the two red peroxidase-peroxide compounds previously described, Theorell discovered a third enzyme-substrate compound which is of greenish colour and shows an absorption band at about 652 mμ. (Theorell, 1941). Since this green compound is the first enzyme-substrate complex formed, the numbering of these derivatives has been modified accordingly. Thus the first green, the first and second red compounds are now referred to as peroxidase-peroxide compounds I, II and III respectively. Theorell (1940) was also able to split peroxidase into its protein and haematin components and to reconstruct active enzymes from the native protein and different haematins (Theorell, Bergström & Åkeson, 1943).

In the present paper a description will be given of a comparatively simple method of preparation of pure horse-radish peroxidase. The spectrophotometric curves of peroxidase and its known reversible derivatives will be given both for the visible and the Soret region, and the properties of peroxidase will be compared with those of catalase and methaemoglobin.

Nomenclature. The catalytic system in which peroxidase is involved is composed of (1) enzyme: peroxidase, (2) substrate: hydrogen peroxide, and (3) a substance undergoing peroxidatic oxidation, usually called an 'acceptor', but which will be referred to in this paper as a hydrogen donor.

EXPERIMENTAL

Methods

Activity of peroxidase preparations was determined through the purpurogallin test as carried out by Elliott & Keilin (1934), Keilin & Mann (1937) and others, which is a slight modification of the original method devised by Willstätter & Stoll (1918). Suitably diluted enzyme (0·1–0·5 ml.) was added to 500 ml. glass-distilled water at 20° containing 1·25 g. resublimed pyrogallol. No commercially available samples of pyrogallol were satisfactory unless resublimed under reduced pressure (approx. 40 mm. Hg). H_2O_2 (0·4 ml. 6% w/v) was added with stirring, and after exactly 5 min. the reaction was stopped by the addition of 5 ml. 5 N-H_2SO_4. The yellow purpurogallin was extracted by shaking with three lots of ether (80, 30, 30 ml.) which had been freed from peroxide by distillation over $FeSO_4 + CaO$. The volume of the ether solution was measured, the solution dried with anhydrous Na_2SO_4 and the concentration of purpurogallin estimated in the Hilger Spekker absorptiometer using 1 cm. cells and Ilford 602 filters. The instrument was calibrated with ethereal solutions of twice recrystallized purpurogallin. The quantity of enzyme taken for the test should be such that less than 10 mg. purpurogallin is produced.

Enzyme activity is expressed in terms of purpurogallin number, P.N. $= w/vd$, where w = mg. purpurogallin formed under the above conditions from v ml. of enzyme solution and d = the dry weight in mg. of 1 ml. of solution.

The number of enzyme units in a peroxidase preparation, E.U. $= Vw/1000v$, where V is the total volume of the peroxidase solution.

Dry weight. Solution (1–2 ml.) was evaporated to constant weight at 105° in a weighing bottle. Prolonged heating was sometimes necessary and the dry residues were often hygroscopic.

Nitrogen was estimated by the micro-Kjeldahl method, the NH_3 being distilled in the Markham (1942) apparatus into HCl which was back titrated with 0·01 N-$Ba(OH)_2$.

Haemin concentration was estimated as follows: A suitable volume (1–2 ml.) of the preparation was pipetted into a 10 ml. volumetric flask containing 0·4 ml. N-NaOH to which 2 ml. pyridine was then added followed by about 50 mg. $Na_2S_2O_4$. The flask was made up to the mark with distilled water, stoppered, well mixed and allowed to stand 0·5 hr. The concentration of haemin, as pyridine haemochromogen, was measured in the Spekker absorptiometer using 1 cm. cells and Ilford 605 filters. The blank cells contained all ingredients except peroxidase. For calibrating the intrument haemin was recrystallized from pyridine-$CHCl_3$-acetic acid. A stock 0·1% solution of this haemin was made up in 0·1 N-NaOH and measured volumes of it were taken for preparation of standard haemochromogens. The final concentration of haemin must not exceed 4 mg./100 ml. The estimation of haemochromogen by spectrophotometric measurement of the height of the sharp α-band at 556 mμ. was considered to be insufficiently reliable since a slight shift of the band can introduce a large error. An additional error may arise from variation in the height of the α-band according to whether protein is present or not. The measurement with a Spekker absorptiometer as described above obviates these difficulties.

Throughout this paper results are expressed as '% haemin', i.e. ferric protoporphyrin chloride, since this compound is weighed out to make the standards. In earlier papers results have been given as '% haematin' (ferric porphyrin hydroxide, formed on dissolving haemin in alkali), although in fact the analyses were based on haemin. Since in peroxidase one of the two residual valencies of Fe is linked to the protein and the other either to hydroxyl (Theorell & Paul, 1944) or to a molecule of water (Keilin & Hartree, 1949), the molecular weights of haemin and of the prosthetic group of peroxidase are 652 and 635–636 respectively. Hence haemin values will be approx. 3% higher than the true values for the accepted prosthetic group. However, for the sake of consistency we shall refer to 'haemin content'.

Spectroscopic observations were carried out with the Zeiss microspectroscope and Hartridge reversion spectroscope.

Absorption curves of peroxidase and its derivatives were determined with the Beckman photoelectric spectrophotometer using 1 cm. cells. The following units are used in plotting the curves: Optical density $D = \log_{10}(I_0/I)$; extinction coefficient $K = D/l$; molar extinction coefficient $\epsilon = D/lc$, where l = optical depth in cm. and c = molar concentration of haemin. In an earlier paper (Keilin & Hartree, 1945*a*) we plotted the absorption constant $\beta = \log_n(I_0/I)/lc$, where c is expressed as g.mol./ml. The absorption coefficient is therefore 2303 times greater in terms of β than in terms of ϵ.

Estimation of peroxidase in horse-radish roots and crude enzyme extracts was carried out by means of a microspectroscope ocular and double wedge trough as described by Elliott & Keilin (1934) and illustrated by Keilin & Wang (1946). The principle of the method consists in matching the absorption bands of pyridine haemochromogen obtained from peroxidase against pyridine haemochromogen prepared from crystalline haemin, or in matching the absorption bands of one of the reversible derivatives (i.e. fluoride) of peroxidase in slices or extracts with those of the corresponding derivative obtained from pure peroxidase.

Calcium phosphate gel. To a 10 l. glass jar containing 1 l. tap water and 250 ml. 0·6 M-$CaCl_2$ was added, with stirring, 250 ml. 0·4 M-Na_3PO_4 followed by sufficient N-acetic acid to bring the pH to about 7·3. The jar was filled with water and the precipitate washed six times by decantation. It was finally collected in centrifuge tubes and suspended in tap water to give about 400 ml. of a thick cream.

Catalase was prepared from horse liver as previously described (Keilin & Hartree, 1945*a*).

Notatin was the same material as described in a previous publication (Keilin & Hartree, 1945*b*).

Alternative procedures. For the estimation of purpurogallin concentration the Spekker absorptiometer can be replaced by the King photoelectric colorimeter (with blue filter) or by any standard visual colorimeter, while the King instrument with the green filter gives very reliable results in the estimation of haematin by the above method. Since it is in the visible rather than in the Soret region of the spectrum that the reactions of peroxidase can be followed, a visual spectrophotometer (e.g. Hilger-Nutting) is adequate for quantitative studies of peroxidase.

Isolation of peroxidase from horse-radish root

Preparation of crude extract. Roots of wild horse-radish (*Cochlearia armoracia* L.) were gathered in spring at the time when the first shoots are appearing and the interference by root polysaccharides during fractionation is at a minimum. Well washed roots, after removal of damaged portions, were cut into 2–4 cm. long sections and passed through a mechanical mincer. The juice was extracted from 50 kg. mince with a stainless steel hydraulic press and water, equal in volume to the press-juice, was well mixed with the mince which was then pressed out again. The process was repeated once more and the three extracts were mixed. When dealing with large quantities of root it is advisable to work in the open air, or alternatively to use a respirator for protection against the strongly lachrymatory mustard oil (allyl *iso*thiocyanate) which, during the mincing, is rapidly liberated from the glucoside sinigrin by a specific glucosidase.

First fractionation. About 69 l. of the above crude extract was treated with 47 kg. $(NH_4)_2SO_4$ and the mixture (0·92 saturation) left standing overnight. Next day the precipitate which had risen to the top was collected, placed on two 27 cm. Büchner funnels containing a bed of kieselguhr and the residual fluid was sucked off. The hard cakes were inverted on to sheets of paper, the kieselguhr scraped off and the precipitate suspended in 2 l. water. Since a suspension can only be obtained by kneading with the fingers it is advisable, on account of the residual mustard oil, to wear rubber gloves and to work in a well ventilated fume cupboard. The suspension was placed in cellophan tubing and dialysed for 3 days against running tap water to remove $(NH_4)_2SO_4$. The contents of the tubing were then filtered to give 4 l. of solution *A*. This solution was treated with 1248 g. $(NH_4)_2SO_4$ (0·5 saturation) and the precipitate (*b*) which was formed only after several hours was filtered off. The filtrate (4600 ml.) was brought to 0·85 saturation by addition of 1150 g. $(NH_4)_2SO_4$ when a sticky precipitate (*c*) appeared gradually and adhered to the glass container. The two precipitates *b* and *c* were separately dissolved in water and dialysed until salt-free, giving respectively the solutions *B* and *C*. Solution *B* contained much coloured impurity, which gave it a greyish-green colour, and had a P.N. of 24. The bulk of the enzyme was in the brown solution *C* which had a P.N. of 129 and contained 2040 E.U. At this stage the fractionation was interrupted while a second batch of horse-radish root (48 kg.) was worked up in the same way to give the corresponding solutions B_1 (P.N. = 32) and C_1 (P.N. = 112; 2100 E.U.).

Further purification of fractions B and B_1. These fractions, which contained 15% of the total enzyme units, were combined to give 1025 ml. solution, treated with 100 ml. tricalcium phosphate gel and centrifuged after 5 min. The supernatant fluid was treated with a further 100 ml. calcium phosphate gel and the gel cakes were discarded. The solution (1050 ml.) was brought to 0·40 saturation by addition of 264 g. $(NH_4)_2SO_4$ after which the precipitate was centrifuged off and discarded since it contained only 89 E.U. of P.N. = 27. A second precipitate obtained by addition of 232 g. $(NH_4)_2SO_4$ (0·70 saturation) was collected on a filter, dissolved in the minimum amount of water and dialysed until completely free of salts. The solution B_2 thus obtained had P.N. = 163 and E.U. = 568.

Isolation of the pure enzyme

The solutions *C*, C_1 and B_2 were now combined to give 2 l. solution *D* (P.N. = 125; E.U. = 4630), the starting material for the next stages of purification.

At all subsequent stages, unless otherwise stated, dialysis was carried out first against running tap water and finally against distilled water. On all occasions where ethanol was present, the liquids and centrifuge tubes were cooled in ice, but the use of a refrigerated centrifuge was found to be unnecessary.

(*a*) *Precipitation with ethanol.* One-third of solution *D* (i.e. 667 ml.) was divided equally among four 1 l. centrifuge tubes to each of which was added 1·5 vol. (250 ml.) 90% ethanol. The tubes were centrifuged for 10 min., the sticky solid was discarded, while the supernatant from each tube was poured into another similar centrifuge tube containing 416 ml. 90% ethanol and 10 ml. 0·6 M-$CaCl_2$; the final concentration of ethanol was thus about 72%. The tubes were immediately centrifuged for 15 min. and the supernatant fluid discarded. The precipitates from the four tubes were suspended in a little distilled water and kept at 0° while the remainder of solution *D* was treated in two lots in the same way. The suspensions of precipitates were finally united and

dialysed overnight against 5 l. distilled water at 5° to give 567 ml. solution *E* (P.N. = 586; E.U. = 4000).

(*b*) *Fractionation with calcium phosphate and ethanol.* Solution *E* was added with stirring to a mixture of 510 ml. water and 76·5 ml. calcium phosphate (37·4 mg./ml.). The calcium phosphate was centrifuged off, washed with 100 ml. water and the cake eventually discarded. (Elution of the cake with 2% potassium oxalate gave a dark greyish solution containing only 56 E.U.) The supernatant solution and washings (1147 ml.) were diluted with an equal volume of 90% ethanol followed by 408 ml. ethanolic calcium phosphate (a mixture of equal volumes of 90% ethanol and the calcium phosphate suspension). The mixture was centrifuged for 10 min. and the cake was immediately suspended in water to a final volume of 200 ml. The supernatant fluid was further treated with two successive lots of ethanolic calcium phosphate (200, 100 ml.) and the practically colourless supernatant was discarded.

(*c*) *Fractionation with* $(NH_4)_2SO_4$. The combined brown calcium phosphate suspensions (500 ml.) were mixed with an equal volume of saturated $(NH_4)_2SO_4$, allowed to stand 1 hr. and centrifuged. The cakes were extracted twice more with small quantities of 0·5 saturated $(NH_4)_2SO_4$ and the washings added to the dark-brown supernatant fluid. The total volume of solution at this stage was 1290 ml. By successive additions of solid $(NH_4)_2SO_4$ to 0·60 saturation (84 g.), 0·68 saturation (68 g.) and 0·85 saturation (171 g.) three precipitates were obtained and filtered off. A little peroxidase was held in solution by the ethanol present even at 0·85 saturation with $(NH_4)_2SO_4$, but by keeping this solution in an open dish overnight a fourth precipitate, containing the remaining enzyme, was obtained.

The first precipitate was rejected, the second was dissolved in water and dialysed to give 222 ml. solution *F* (P.N. = 753; 579 E.U.) while the third and fourth were combined, dissolved and dialysed to give 232 ml. solution *G* (P.N. = 831; 3520 E.U.).

(*d*) *Heat denaturation of impurities followed by* $(NH_4)_2SO_4$ *fractionation.* Solution *G* was diluted with water to 1334 ml. and treated with 306 g. $(NH_4)_2SO_4$ (0·38 saturation). Quantities of $\geqslant$ 200 ml. were heated rapidly to 62°, held at this temperature for 10 min. and rapidly cooled to room temperature. The combined heat-treated solutions were centrifuged to give a grey cake insoluble in water, and a slightly cloudy fluid which was clarified by filtration through Whatman no. 5 paper. (Although centrifuging did not yield a clear fluid, it accelerated the subsequent filtration.) The clear fluid (1340 ml.) was fractionated by successive addition of $(NH_4)_2SO_4$: 168 g. (0·56 saturation), 75 g. (0·65 saturation) and 44 g. (0·70 saturation). The three precipitates were filtered off, dissolved separately in a little water and dialysed to give solutions containing 700, 2550 and 344 E.U. respectively. The second fraction, *H* (0·65–0·70 saturation), had a total volume of 104 ml. All attempts to increase the P.N. and haemin content of fraction *H* by further $(NH_4)_2SO_4$ fractionation were unsuccessful. In fact, the variations between different fractions were within experimental error and the average analytical figures were: haemin 1·61%, nitrogen 13·2%, P.N. = 1220 ± 50. The behaviour of fraction *H* in the ultracentrifuge showed that it was almost homogeneous (see Addendum to this paper, p. 105).

Absorption curves of peroxidase and its derivatives

The positions of the absorption bands of peroxidase and its derivatives have been recorded by Keilin & Mann (1937). The spectrophotometric curves of free peroxidase and 'paraperoxidase' have been given by Theorell (1941, 1942*a*), whereas curves for the Soret bands of the three peroxidase-peroxide compounds and of the peroxidase-fluoride and peroxidase-cyanide compounds have been recorded by Chance (1949*b*).

For measurements of light absorption of peroxidase and its derivatives in the Beckman spectrophotometer, solutions were prepared as shown in Table 1. They were suitable for density determinations at the red end of the spectrum while at lower wavelengths the peroxidase concentration had to be reduced five to twenty times. In making suitable dilutions the concentrations of the other ingredients were kept constant. The absorption curves of peroxidase and of its derivatives except those with H_2O_2 are grouped in two figures. Fig. 1 represents the absorption curves of free peroxidase and of its compounds with fluoride, cyanide and azide, while Fig. 2 shows the absorption of alkaline peroxidase, of peroxidase reduced with $Na_2S_2O_4$ and of the latter after treatment with CO.

The comparison of these curves with those of the corresponding derivatives of methaemoglobin and

Table 1. *Preparation of solutions for spectrophotometric measurements on peroxidase and various derivatives other than the* H_2O_2 *compounds*

(A stock solution of peroxidase (0·386 mM, P.N. = 1220) was used to prepare the following solutions.)

Peroxidase	Other ingredients	Final haemin concn. (μM)	Spectrum obtained
a 2·0 ml. stock	8 ml. 0·25 M-Phosphate buffer, pH 5·6	77·4	Peroxidase
b 3·0 ml., *a*	0·3 ml. 0·7 M-KCN in M-KH_2PO_4	70·3	CN^--peroxidase
c 3·0 ml., *a*	0·6 ml. 2·5 M-NaF	64·5	F^--peroxidase
d 1·0 ml. stock	4·0 ml. 0·7 M-NaN_3 in 0·25 M-acetate buffer, pH 4·25	77·4	N_3^--peroxidase
e 0·6 ml. stock	3·0 ml. 0·6 M-K_2CO_3 (pH 11·4)	64·5	Alkaline peroxidase
f 0·5 ml. stock	4·0 ml. 0·25 M-Phosphate buffer, pH 6·8, 20 mg. $Na_2S_2O_4$*	42·9	Reduced peroxidase
g as *f*	Saturated with CO*	42·9	CO reduced peroxidase

* Solutions covered with layer of paraffin during measurements.

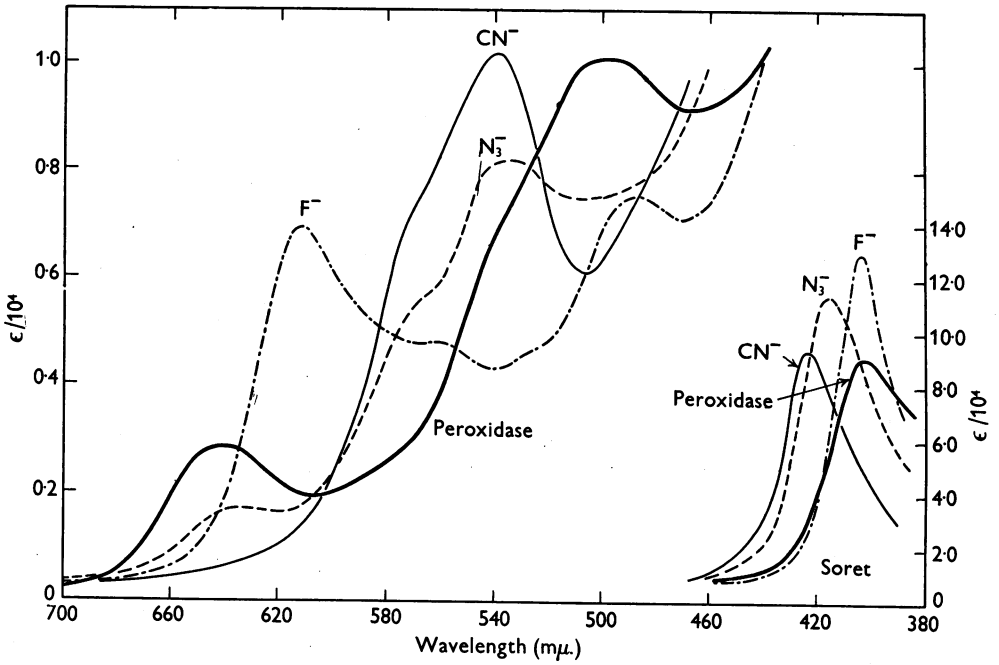

Fig. 1. Absolute absorption spectra of peroxidase and of its fluoride (F^-), cyanide (CN^-) and azide (N_3^-) compounds. For details of solutions used see Table 1. $\epsilon = 1/cl \log_{10} I_0/I$, where c = molar concentration of haematin and l = optical depth in cm. Ordinate scale ($\epsilon/10^4$) reduced 20-fold for Soret bands.

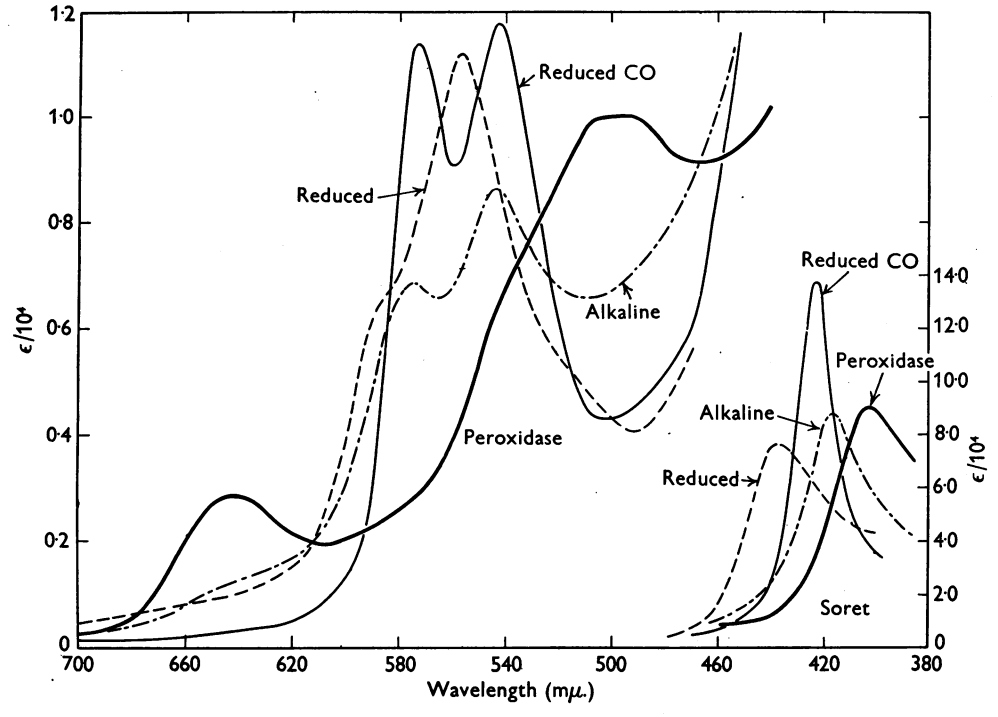

Fig. 2. Absolute absorption spectra of peroxidase, alkaline peroxidase, reduced peroxidase and CO-reduced peroxidase. For details of solution used see Table 1. For definition of units see Fig. 1.

catalase will be discussed at a later stage. Here we shall note only a few characteristic features of the peroxidase curves.

(1) The height of the band in the red of free peroxidase is somewhat greater than that given by Theorell & Paul (1944). Their value for $\beta_{640} = 0{\cdot}585 \times 10^7$ is equivalent to $\epsilon_{640} = 0{\cdot}254 \times 10^4$, whereas our value is $0{\cdot}284 \times 10^4$. A similar proportional increase in the value of ϵ for the Soret band was found on comparing our results (see Fig. 3) with those of Chance (1949*b*).

(2) The azide derivative of peroxidase is only formed below pH 4·5 (Theorell, 1942*c*), while at pH lower than 4 the compound becomes unstable as indicated by a drift in optical density readings.

detection and estimation of peroxidase in roots and in crude extracts is more easily carried out after its transformation to one of these derivatives.

(5) The Soret band of CO-ferroperoxidase is much higher and sharper than the corresponding band in other derivatives of peroxidase.

Peroxidase-peroxide compounds

Peroxidase, as was mentioned previously, forms three compounds with H_2O_2: the green compound I of Theorell (1941) which is a precursor of the two red compounds II and III of Keilin & Mann (1937). Compound II, which shows absorption bands at 555 and 527 mμ., is formed with as little as 1 mol. H_2O_2

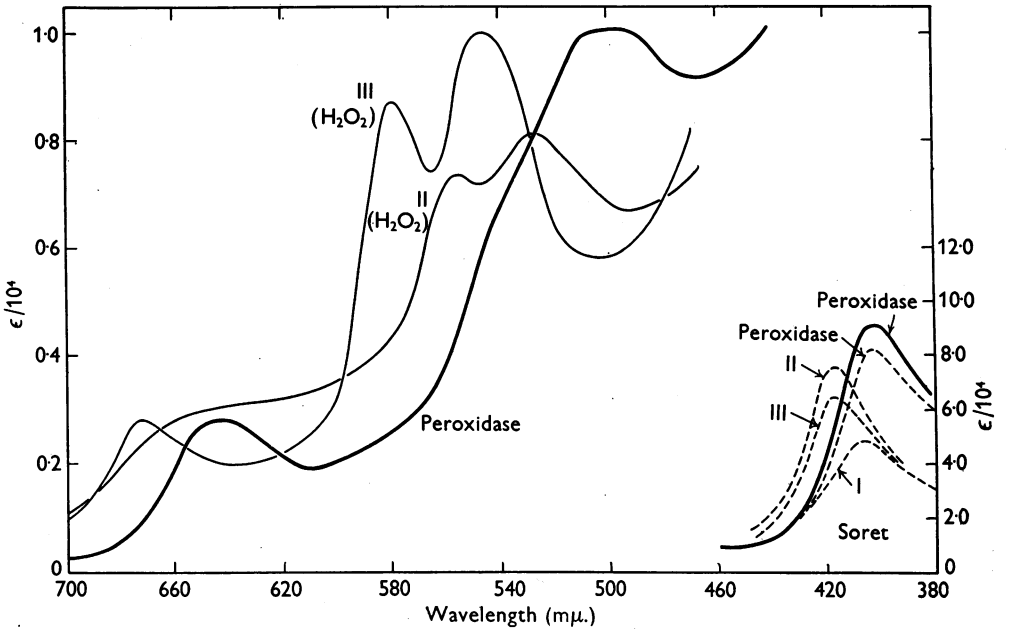

Fig. 3. Absolute absorption spectra of peroxidase and peroxidase-H_2O_2 compounds I, II and III. The pecked lines represent the Soret bands of peroxidase and of its three peroxide complexes as obtained by Chance (1949*b*). The full lines are based on the results of the present investigation.

(3) On addition of fluoride to peroxidase the absorption band in the red is replaced by a much stronger and sharper band at 612 mμ. which is, however, not as strong as that of the cyanide compound or of reduced peroxidase and its CO derivative. Nevertheless, peroxidase in horse-radish roots is more readily detected as the fluoride compound than as any other derivative since the main band is at a wavelength where there is no interference from other pigments and, at the same time, it is more intense than the peroxidase band at 641 mμ. The Soret bands of the F^- and CN^- compounds have also been plotted by Chance (1949*b*, Fig. 6).

(4) Since all derivatives of peroxidase show much sharper absorption bands than the free enzyme, the per Fe atom and even in absence of acceptor it rapidly reverts to free peroxidase. On the other hand, the addition of 15–25 molecules of H_2O_2 leads to the formation of compound III which is also red and shows absorption bands at 583 and 546 mμ. Furthermore, a peroxide concentration high enough to yield 100% of complex III will bring about a fairly rapid destruction of the enzyme with an intermediate formation of a compound showing a distinct band in the red at 670 mμ. This compound also appears when any of the peroxidase-peroxide complexes (I, II or III) are repeatedly reformed by successive additions of small amounts of H_2O_2 to peroxidase liberated from the complex. The compound with an absorption band at 670 mμ. resembles

the complex IV described by Chance (1949*b*) as being formed in the reaction between peroxidase and methyl hydrogen peroxide where, in absence of a derivative corresponding to peroxidase-H_2O_2 III, it follows directly the formation of complexes I and II. It is, however, quite conceivable that the band at 670 mμ. belongs to a peroxidase with a modified prosthetic group (a verdo-type as was suggested by Chance) not necessarily combined with peroxide. This complex therefore is not comparable with I, II and III and the term complex IV is hardly appropriate for it.

Spectrophotometric curves of the peroxidase-peroxide complexes (Fig. 3) were determined by us only for complexes II and III in the visible region. Since the light absorption is constantly changing during these determinations, it is necessary to obtain records of changes of density with time at each of a number of wavelengths and from such records to construct the curves. Under the experimental conditions of these determinations no oxygen bubbles appeared. The Soret bands of all three complexes are taken from a recent paper by Chance (1949*b*).

Peroxidase-peroxide I is the first compound formed when a very small amount of H_2O_2 is added to peroxidase. Direct spectroscopic examination reveals a band at 657 mμ. As the compound is very unstable and rapidly passes into complex II its spectrophotometric curve cannot be recorded by the present method. However, its Soret band, determined by Chance (1949*a*, *b*) using his rapid flow method, is given in Fig. 3.

Peroxidase-peroxide II. The stock peroxidase solution, 4·52 ml., described in Table 1, was diluted with 0·25 M-phosphate buffer, pH 5·6, to 25 ml. and a solution of H_2O_2 (0·00446 M) was made up in 0·125 M-phosphate buffer, pH 5·6. The former solution (2·5 ml.) was mixed with 1 ml. of the latter, the mixture placed immediately in a Beckman absorption cell and readings of density (*D*) were taken with the minimum delay at 595 and 586 mμ. Records were kept of the times at which each reading was taken, and six further concurrent sets of readings were then taken alternately at the same two wavelengths. Thus during 15 min. seven values of *D* at definite times after mixing were obtained at each of these wavelengths. The above operations were repeated at seven other series of two wavelengths so that data became available for each of the following sixteen wavelengths: 595, 586, 583, 580, 578, 573, 568, 564, 558, 553, 545, 538, 530, 520, 500 and 470 mμ. In the region above 595 mμ. the changes of *D* were slower and it was possible to 'scan' the following wavelengths using one peroxidase sample: 750, 700, 680, 672, 660, 640 and 615 mμ. From the various density-time curves, absorption curves at various times were constructed and those for 1·5, 7, 10 and 13·5 min. are given in Fig. 4. The 1·5 min. curve represents a mixture of complexes II and III in which the latter predominates. Since the H_2O_2 slowly decomposes the later curves show the α- and β-bands of II. The ratio of the band heights approached a constant value at 13·5 min. and the corresponding curve may therefore be taken as the nearest approximation to that of peroxidase-peroxide II (see also Fig. 3).

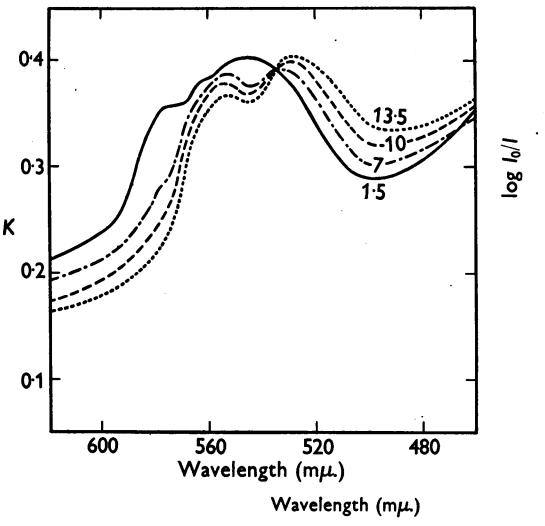

Fig. 4. Changes of absorption spectrum of peroxidase with time (min.) after addition of H_2O_2. Series II experiments. Peroxidase, $5{\cdot}00 \times 10^{-5}$ M; H_2O_2, $1{\cdot}28 \times 10^{-3}$ M; 0·2 M-phosphate buffer, pH 5·6. $K = 1/l \log_{10} I_0/I$, where l = optical depth (1 cm.).

Peroxidase-peroxide III. For the study of this complex the same solutions were used as for complex II except that the H_2O_2 concentration was five times greater. Since *D* now changes more slowly each peroxidase sample could be used for the study of the density-time changes at four wavelengths instead of two. The region 595–750 mμ., as in the previous experiment, could be covered with one sample of enzyme. It was found that there was a drift in readings during the first minute after which the absorption remained constant until the end of the third minute. Subsequently there was a progressive decrease in *D* at all wavelengths indicating a destruction of peroxidase. The 3 min. absorption curve, which may be taken as a close approximation to that of complex III, is shown in Fig. 3. Since a slight decomposition of the pigment has occurred the bands as represented are a little too low.

Of the three complexes the peroxidase-peroxide III shows the most distinct absorption spectrum with two bands in the visible region resembling those of methaemoglobin-peroxide. The absorption spectrum of complex II is of similar pattern, but the

bands are less distinct and nearer to the blue. On the other hand, the Soret band of III is lower than that of II. The absorption spectrum of I is of the same pattern as that of free peroxidase, but it has an exceptionally low Soret band (Fig. 3).

A summary of the absorption maxima of different derivatives, based upon the spectrophotometric curves represented in Figs. 1–3, is given in Table 2.

Table 2. *Wavelengths (mμ.) of absorption maxima and (in parentheses) the corresponding values of* $\epsilon \times 10^{-4}$ *for peroxidase and derivatives*

Peroxidase (Fe^{+++})	641·5 (0·284)	497 (1·003)	403 (9·10)
Alkaline peroxidase	574·5 (0·686)	545 (0·864)	416 (8·85)
Reduced peroxidase (Fe^{++})	556 (1·122)	—	437 (7·73)
Reduced peroxidase CO	572·5 (1·140)	542 (1·177)	423 (13·80)
CN^--peroxidase	538 (1·020)	—	423 (9·39)
F^--peroxidase	612 (0·693)	487·5 (0·755)	404 (13·00)
N_3^--peroxidase	635 (0·170)	534 (0·820)	416 (11·40)

The wavelengths of the absorption bands of the unstable H_2O_2 complexes recorded in Figs. 3, 4 and 6 differ slightly from the figures obtained on direct visual observation with either the microspectroscope or reversion spectroscope (Table 3). This is due partly to the differing experimental conditions and partly to the fact that close agreement between the two methods can only be obtained when the absorption bands are symmetrical.

Effect of low temperature on the absorption spectrum of peroxidase

On freezing and cooling to the temperature of liquid air, the absorption bands of peroxidase and its derivatives are sharpened, greatly intensified and slightly shifted towards the blue end of the spectrum. In this respect peroxidase behaves like some other derivatives of haematin and many other substances showing selective absorption (Keilin & Hartree, 1949, 1950*a*). Freezing in liquid air also makes possible the direct observation in horse-radish root slices of the absorption band in the red of free peroxidase which, owing to its diffuse absorption spectrum, is hardly perceptible at ordinary temperatures.

As already mentioned, peroxidase, like methaemoglobin, shows very marked changes in colour and absorption spectrum with changes in pH. Thus at pH above 11 a reddish alkaline form of peroxidase is formed with bands at 574 and 545 mμ. (Fig. 2). When a neutral solution of peroxidase is cooled its absorption band in the red is intensified and shifted 7–9 mμ. nearer the blue end of the spectrum. On the other hand, when alkaline peroxidase is cooled, its colour and absorption spectrum revert to those of neutral peroxidase with the appearance of the characteristic band in the red. On warming the solution to room temperature the colour and absorption spectrum of alkaline peroxidase rapidly reappear. We observed a precisely similar change on cooling a solution of alkaline methaemoglobin and interpreted it on the assumption that the sixth octahedral position around the iron atom in acid methaemoglobin and also in neutral peroxidase is occupied by a molecule of water. The transition to the alkaline form would then involve not the addition of a hydroxyl ion but rather the dissociation of a proton, as was previously suggested by Coryell, Stitt & Pauling (1937) in the case of methaemoglobin. The shift in equilibrium observed on cooling alkaline peroxidase or alkaline methaemoglobin could thus be ascribed to a suppression of ionization. This is strongly supported by the fact that similar changes in colour can be observed on cooling solutions of some indicators (Keilin & Hartree, 1949). Below a certain temperature cooling affects the colour of indicators by suppressing their ionization, a phenomenon which is general for electrolytes (Harned & Embree, 1934). The spectroscopic study of peroxidase at low temperatures does not support the view put forward by Theorell & Paul (1944) that between pH 5 and 11 the sixth co-ordination place is occupied by a hydroxyl group while at pH 11 two hydroxyl groups are attached to the iron atom by covalent links.

On the so-called 'paraperoxidase'

It was claimed at one time by Theorell (1940, 1942*a*) that horse-radish roots contain two different peroxidase enzymes: peroxidase I or paraperoxidase and peroxidase II or true peroxidase. According to Theorell the characteristic properties of these peroxidases are as follows:

Peroxidase I or paraperoxidase is present in horse-radish roots in variable but always much lower concentrations than those of true peroxidase. It shows an absorption spectrum of the parahaematin type with two bands at 583 and 548 mμ. which on reduction with $Na_2S_2O_4$ are replaced by typical haemochromogen bands lying at 565 and 535 mμ. Peroxidase I is precipitated with picric acid and on cataphoresis at pH 7·5 it moves towards the cathode. Its peroxidatic activity on a haemin basis is of the same magnitude as that of true peroxidase. It catalyses the oxidation of dihydroxymaleic acid directly and this reaction is almost completely inhibited by 10^{-5} M-KCN.

Peroxidase II or true peroxidase shows a methaemoglobin-like spectrum with bands at 640 and

498 mμ.; it is not precipitated by picric acid and on cataphoresis at pH 7·5 it moves towards the anode. For oxidation of dihydroxymaleic acid it requires a carrier such as quinol and this reaction is not affected by cyanide.

These views were extended by Theorell (1942*a*) in his main paper on the subject and in a general review (Theorell, 1943). It was, however, partly withdrawn by Theorell & Åkeson (1942) in a paper dealing with the microdetermination of amino-acids in peroxidase and catalase. Unfortunately this withdrawal was lost in a paper the subject matter of which had no direct bearing on the problem, with the result that the supposed existence in horse-radish roots of two peroxidases has reached books and reviews. Moreover, even in his more recent review, Theorell (1947) considers the so-called paraperoxidase as only 'possibly a derivative of peroxidase' and what is important, in none of his papers or reviews does he refer to his earlier statement that true peroxidase is unable to catalyse directly the aerobic oxidation of dihydroxymaleic acid.

Personal observations. In our experience with many preparations of horse-radish peroxidase we have always obtained only a typical true peroxidase with the characteristic methaemoglobin-like absorption spectrum and even the most purified fractions catalysed the oxidation of dihydroxymaleic acid without the addition of quinol. However, on several occasions we found that when a solution of highly purified true peroxidase was left for a considerable time in an ice chest its methaemoglobin-like absorption spectrum turned gradually into that of a parahaematin. The paraperoxidase thus obtained showed the following properties:

(1) Instead of the normal brown colour the solution was reddish-brown and showed two absorption bands in the visible region lying at about 582 and 543 mμ.

(2) On addition of $Na_2S_2O_4$ a haemochromogen-like spectrum rapidly appeared but with the bands lying nearer to the red end of the spectrum than usual, i.e. at about 565 and 535 mμ. This spectrum was, however, soon replaced by that of typical reduced true peroxidase with a wide, strong band at 556 and a narrow, faint band at 594 mμ.

(3) On addition of KH_2PO_4 and NaF the solution turned greenish and the band of fluoride peroxidase appeared at 612 mμ. while the bands of parahaematin disappeared.

(4) On addition of KCN the solution turned red and showed the characteristic cyan-peroxidase absorption spectrum.

(5) On addition of H_2O_2 the solution showed in turn the absorption spectra of peroxidase-peroxide compounds II and III.

(6) The peroxidatic activity of the solution when the parahaematin spectrum was fully developed was only 38 % lower than that of the original peroxidase solution.

All this clearly shows that paraperoxidase is a transformation product of true peroxidase. What is important, however, is that both compounds react with fluoride, cyanide and a reducer ($Na_2S_2O_4$) to yield similar derivatives. It is therefore conceivable that paraperoxidase can, under certain conditions, revert to the original product and that its formation is due to a partial and reversible denaturation of the protein. Should this be the case, then the change to paraperoxidase is in all probability due to a replacement of the water molecule occupying the sixth co-ordination position by a group in the protein, so that in paraperoxidase two of the six iron valencies are satisfied by the protein. Although paraperoxidase is not a natural product, its properties and the conditions of its formation deserve further study. The term paraperoxidase should therefore be retained for this interesting derivative.

In this connexion, it is of interest to note that similar spectroscopic changes may also occur in solutions of catalase. Thus a solution of horse-liver catalase of Kat.f. = 43,000 was left in a refrigerator for about 2 weeks during which time its activity remained constant. At the end of this period, however, its absorption was considerably modified, the band in the red at 622 mμ. being greatly diminished in intensity while two strong bands appeared at 575 and 537 mμ. This absorption spectrum was not affected on treating the solution with either $Na_2S_2O_4$ or $K_3Fe(CN)_6$, but on addition of NaN_3 the two new bands disappeared and a typical azide-catalase spectrum was formed. On passing a current of air through the solution of modified catalase for several hours the absorption spectrum reverted to that of normal catalase. So far no explanation can be offered as to the nature of these changes in catalase solutions.

Reaction of catalase with hydrogen peroxide

Peroxidatic activity of catalase. The rapid decomposition of H_2O_2 by tissue extracts has been known for more than a century (Thenard, 1818). It was considered at first as a general property of tissues shared only with finely divided metals and later as a property common to all enzymes. It was eventually recognized by several workers and definitely demonstrated by Loew (1901) that this reaction is catalysed by one specific enzyme which he named *catalase*. From that time the decomposition of H_2O_2 to molecular oxygen and water was believed to be the sole function of catalase. However, we have shown (Keilin & Hartree, 1936*b*) that in addition to this reaction catalase is also capable of catalysing the coupled oxidation of the lower alcohols by means of H_2O_2 produced in certain primary oxidation reactions (e.g. those catalysed by xanthine oxidase,

uricase, D-amino-acid oxidase and glucose oxidase). Although for this reaction catalase can also use free H_2O_2, provided it is added slowly and continuously, yet the primary oxidation reaction is by far the most efficient mechanism of supply of H_2O_2 since nearly every molecule of H_2O_2 thus formed can be utilized in the oxidation of alcohols. In all other methods of supply of H_2O_2 only a small fraction is utilized in peroxidatic oxidation, the main portion being decomposed catalatically (Keilin & Hartree, 1945*b*). These observations confirmed our earlier conclusion that 'the physiological function of catalase would be mainly peroxidatic and only in exceptional cases a catalatic one' (Keilin & Hartree, 1936*b*). The peroxidatic activity of catalase has been confirmed and extended by other workers. To the six alcohols: methanol, ethanol, *n*-propanol, *iso*butanol, β-aminoethanol and ethylene glycol, which we previously found acting as hydrogen donors in peroxidatic oxidations catalysed by catalase, Chance (1947, 1948) has added formaldehyde, formic acid, ascorbic acid and peroxides, while Heppel & Porterfield (1949) have added nitrite. What is, however, of special interst is that among hydrogen donors in peroxidatic oxidation by catalase Chance (1948, 1949*d*) includes H_2O_2 itself. In other words, H_2O_2 may act both as substrate and hydrogen donor, the reaction consisting in the oxidation of one molecule of H_2O_2 by another. This would bring even the catalatic decomposition of H_2O_2 into the category of peroxidatic reactions.

Absorption spectra of catalase-peroxide compounds

We have seen that when H_2O_2 is added to methaemoglobin or peroxidase the colour and the absorption spectrum of these haemoproteins undergo marked changes (cf. pp. 88 and 93). On the other hand, when H_2O_2 is added to catalase, the explosive decomposition of H_2O_2 to O_2 and water is not accompanied by any obvious changes in the colour and absorption spectrum of the solution that could be observed in the usual way. Only by applying the rapid flow method and a very sensitive spectrophotometer to the study of this problem was Chance (1947, 1949*b*) able to observe the formation of the primary catalase-peroxide complex, which can be recognized by a slight decrease and a shift towards the red of the Soret band as well as by the presence of a band at about 670 mμ. (Chance, 1950). He was also able to show that the formation of this complex involved only one of the four haematin nuclei of mammalian catalase. Moreover, he considers that 'the attachment of more than one molecule of hydrogen peroxide to catalase results in an as yet undetected complex which rapidly decomposes'. In other words it is the catalatic activity of the enzyme that limits the saturation value of the primary catalase H_2O_2 complex to 1 molecule of H_2O_2 per molecule of catalase.

With H_2O_2 continuously generated by an oxidizing system such as notatin, glucose and oxygen, Chance (1950) observed that the rapidly formed primary catalase-H_2O_2 complex (I) changes gradually into a secondary complex (II) which shows two absorption bands in the visible region: α, 572 and β, 536 mμ. He has studied the kinetics of formation of complex II under various conditions and has suggested that the compound with two absorption bands at 565 and 535 mμ. which is formed when ascorbic acid is added to catalase is not a catalase-ascorbic complex, as was believed by Lemberg & Foulkes (1948), but is in fact the catalase-peroxide complex II, the H_2O_2 arising from autoxidation of ascorbic acid. The variation in the position of absorption bands of complex II with the experimental conditions led us to further studies of the reactions of catalase with continuously generated H_2O_2. The main result of this work was the characterization of a new complex which can be described as catalase-H_2O_2 III.

Experiments with catalase, notatin and glucose. To a small vertical glass tube, 9 mm. diameter, with a fused optical bottom, was added 0·4 ml. catalase (0·184 mM with respect to haematin), 0·4 ml. 0·2 M-acetate buffer pH 5·0, 0·1 ml. 0·5 M-glucose and a trace of octyl alcohol. The tube was stood above the condenser of a microscope and the reaction followed with a microspectroscope. On adding 0·1 ml. notatin solution (0·1 %) and passing a fine stream of air bubbles through the mixture for a few seconds, the colour of the solution turned from brown to red and the absorption bands of free catalase were replaced by two bands: α 568 and β 536 mμ. The positions of these bands were confirmed with a Hartridge reversion spectroscope. If aeration was continued the α-band tended to spread towards the red and thus become asymmetric, while eventually a very feeble band appeared at 585 mμ. On addition of 0·2 ml. 0·1 N-H_2O_2 to the aerated solution, the absorption bands of complex II were replaced by two bands lying nearer to the red: α, 585 and β, 545 mμ. which we ascribe to the complex III. Both II and III revert on standing to free catalase. Experiments at different pH showed that the above reactions proceeded rapidly only within the range 4·0–5·5.

Experiments with catalase and ascorbic acid. It can easily be demonstrated that the changes in colour and absorption spectrum of catalase that are brought about by ascorbic acid are due to the formation of catalase-H_2O_2 II and not to a compound between catalase and ascorbic acid. Thus the reaction requires the presence of oxygen, it is greatly accelerated by the addition of copper salts and the compound obtained is spectroscopically indistinguishable from that observed on aeration of catalase + notatin + glucose.

The reaction was carried out in the vertical glass tubes using 0·4 ml. catalase (as above), 0·4 ml. buffer, 0·1 ml. neutralized ascorbic acid (0·5 M) and various quantities of $CuSO_4$ (0–1 μmole) in 0·1–0·2 ml. water. In absence of copper, aeration of this mixture within the pH range 3·9–6·8 caused no change in absorption spectrum during 5 min. In an experiment at pH 5·0 (0·1 M-acetate buffer) the addition of 0·1 μmole $CuSO_4$ during aeration had no effect, whereas 0·4 μmole gave rise immediately to complex II with bands at 568 and 536 mμ. When the solution was allowed to stand for a few minutes the α-band widened towards the red and appeared to have a maximum at 572 mμ. At this stage the solution was aerated again and a further 0·6 μmole $CuSO_4$ was added. The complex II was immediately and completely transformed into complex III with bands at 586 and 546 mμ. At pH 3·9 a very similar result was obtained, but more ascorbic acid was required for the complete formation of complex III. Under similar conditions at pH 5 the very sparingly ionized copper glycine gave rise to complex II plus a trace of III while even the water-insoluble copper acetylacetone catalysed the slow production of complex II. In all experiments involving ascorbic acid the formation of complexes with H_2O_2 was accompanied by precipitation of catalase. No precipitate was formed if either ascorbic acid or copper was omitted. Nevertheless, even after precipitation the spectra reverted on standing to that of free catalase.

From the above results it is clear that the absorption bands of catalase-H_2O_2 II are: α, 568 and β, 536 mμ., while those of complex III are: α, 585 and β, 545 mμ. The α-band at 572 mμ. previously recorded (Chance, 1950) is probably that of complex II at a stage where it has undergone a slight conversion to III.

Reaction of methaemoglobin (Methb) with hydrogen peroxide

It has been established (Keilin & Hartree, 1935; Haurowitz, 1935) that methaemoglobin forms with H_2O_2 and with ethyl hydroperoxide well defined compounds analogous to those which it forms with cyanide, fluoride, sulphide and azide. The formation of Methb-peroxide requires 1 molecule of peroxide per iron atom of Methb. The Methb-hydrogen peroxide (abbreviated to Methb-OOH) is red in colour and shows two bands: α, 589 and β, 545 mμ. The β-band is stronger than the α-band which is diffuse, asymmetric and may even appear to have two maxima. The complex nature of this band as well as the fact that both peroxidase and catalase form with H_2O_2 several spectroscopically recognizable compounds have prompted us to re-examine more closely the spectroscopic changes which accompany the reaction between Methb and H_2O_2. A preliminary account of this work has already appeared (Keilin & Hartree, 1950*b*).

For this purpose Methb was obtained from catalase-free crystalline horse oxyhaemoglobin by treatment with $NaNO_2$ or with $K_3Fe(CN)_6$ and subsequent dialysis. The experiments were carried out in the small vertical tubes described above and the reactions were followed with a microspectroscope. The solution of Methb used in these experiments was 0·384 mM with respect to haematin and was made up in 0·2 M-phosphate buffer pH 6·8. A 6 mm. depth of this solution clearly showed the absorption band in the red of Methb. The quantity of H_2O_2 added to Methb will be expressed in terms of the molar ratio (R) of H_2O_2 to haematin. When $R = 1$ the formation of Methb-OOH was 80% complete (see also Keilin & Hartree, 1935). When $R = 2·5$–5, Methb-OOH was completely formed, but on standing it decomposed liberating acid Methb. During this process part of the Methb underwent destruction.

As the concentration of H_2O_2 was increased the double appearance of the α-band became more marked, the band showing two maxima at 578 and 592 mμ. When $R = 25$ the α-band was distinctly double, its two components being of almost equal intensity. When $R = 100$ the band at 578 mμ. was much stronger than the band at 592 mμ. When R reached 250–500 the band at 578 mμ. predominated and after a short time the band at 592 mμ. became completely invisible. Thus in presence of an excess of H_2O_2 the Methb-OOH was replaced by another compound which could easily be recognized as oxyhaemoglobin (HbO_2). In fact its absorption spectrum was indistinguishable from that of HbO_2. On treatment with $Na_2S_2O_4$ it gave deoxygenated haemoglobin (Hb) and in presence of CO it formed HbCO. The same sequence of events can be observed by using the notatin system to supply H_2O_2 as described above for the study of catalase-H_2O_2 compounds. On treating a mixture of Methb, notatin and glucose at pH 6·8 with a stream of air Methb-OOH is rapidly formed, but does not become appreciably reduced to HbO_2. Since at the same time the concentration of H_2O_2 remains very low and there is consequently no appreciable destruction of haematin, the maximum at 592 mμ. stands out very clearly with only a faint shading at 578 mμ. representing a trace of HbO_2.

In experiments with H_2O_2 added as a solution it is of special interest that bubbles of O_2 deriving from the decomposition of H_2O_2 do not appear until the α-band of HbO_2 becomes discernible, and active decomposition of H_2O_2 takes place only when the bands of HbO_2 are already very distinct. The reaction between Methb and H_2O_2 therefore consists in the formation of the Methb-OOH complex which, on further reaction with H_2O_2, brings about its decomposition to molecular O_2 and water, a

reaction which is accompanied by the reduction of Methb to Hb and the oxygenation of the latter to HbO_2. The greater the excess of H_2O_2 added from the beginning the more rapid and complete is the formation of HbO_2 and the more vigorous is the decomposition of H_2O_2. But however great is the excess of H_2O_2, the appearance of HbO_2 is always preceded by a transient stage when the total Methb is converted into Methb-OOH. On addition of a suitable hydrogen donor, such as ascorbic acid, which can be oxidized peroxidatically, to a solution showing the absorption bands of HbO_2 and Methb-OOH, the latter is rapidly decomposed liberating Methb while HbO_2 remains temporarily unchanged. The catalatic decomposition of H_2O_2, together with the appearance of HbO_2, are soon followed by a destruction of the haem nucleus as shown by disappearance of absorption bands of the solution which becomes colourless. Since the absolute extinction of the α-band of HbO_2 is much greater than that of Methb-OOH when the relative concentrations of these two compounds in the solution are equal, the α-band of HbO_2 appears to be much stronger than that of Methb-OOH.

All the above reactions have been observed with both recrystallized methaemoglobin and metmyoglobin at pH's varying between 5·5 and 9·2. At pH below 5 the reaction follows a different course. Thus with great excess of H_2O_2 the Methb-OOH formed is rapidly replaced by HbO_2, whereas with smaller concentration of H_2O_2 the Methb-OOH is gradually replaced by a new compound with a wide absorption band at 590 mμ. and showing red-green dichroism. The formation of this compound is preceded by an intermediate stage devoid of any distinct bands in the visible region. However, the addition of NaN_3 or NaF at this stage yields the corresponding Methb derivative, which can readily be identified spectroscopically, while $Na_2S_2O_4$ gives rise to Hb. The haematin prosthetic group, or at least a considerable proportion of it, is thus intact. If the minimum of H_2O_2 is used the compound showing the 590 mμ. band is stable for an hour or more. These conditions are obtained by adding 0·1 ml. 0·05 N-H_2O_2 to 7·5 ml. Methb (0·077 mM with respect to haematin) in 0·2 M-acetate buffer pH 4·5. The 590 mμ. band reaches full intensity after about 8 min. The addition of pyridine and $Na_2S_2O_4$ gives rise to a strong protohaemochromogen with two very faint bands nearer to the red, but the addition of NaF causes only a slow appearance of fluoride-Methb in very poor yield. These results indicate an oxidation of the porphyrin ring, possibly at the methine bridges, without an opening of the tetrapyrrol structure. This view is confirmed by the finding that the Soret band persists, but is much less intense ($\epsilon^{410}_{max.} = 40{,}000$ as compared with $\epsilon^{405}_{max.} = 163{,}000$ for acid Methb).

Since the peroxidatic and catalatic activities of Methb, although much lower than those of peroxidase and catalase, are nevertheless very distinct, Methb can be considered as a suitable model for the study of the reactions catalysed by these two enzymes. As the catalatic decomposition of H_2O_2 by Methb involves the formation of Methb-OOH and subsequent valency changes of Methb iron, it is reasonable to assume that the similar decomposition of H_2O_2 by catalase is also accompanied by valency changes of catalase iron, a view which is, moreover, supported by the study of azide-catalase (Keilin & Hartree, 1936*a*, 1945*a*).

DISCUSSION

Properties of peroxidase

As the quantitative study of different properties, including the absolute absorption spectra, of peroxidase required highly purified enzyme preparations a comparatively simple method was developed for isolation and purification of peroxidase from horse-radish roots. Our most active preparation had a P.N. of about 1220. Its haemin content was 1·61 % which corresponds to a minimum molecular weight of 40,500 (± 600) while sedimentation-diffusion studies carried out by Dr A. G. Ogston and Dr R. Cecil (see Addendum, p. 105) gave the figure 39,800.

Although the haemin content of our purest peroxidase preparation (1·61 %) is higher than the value (1·48 %) given by Theorell (1942*a*), it should perhaps be mentioned that the haemin content of one of his crystalline preparations, of which he obtained 2·8 g., was actually 1·62 %. He concluded, however, that this preparation 'still contained some hemin that did not belong to the peroxidase molecule, which according to the determination of molecular weight should contain 1·48 % haemin'. The high haemin value (1·62 %) he ascribed to the omission of electrodialysis which should remove the extraneous haematin. The fact that a haemin content of 1·48 % was obtained by Theorell after electrophoresis 'performed at a much too high pH value', which reduced the yield of the enzyme from 2·8 to 0·5 g. suggests the possibility that the enzyme of this preparation may have undergone a change involving loss of haematin.

Although our values of haemin content and of molecular weight differ from those of Theorell by only 8–9 % far greater discrepancies appear in the figure for P.N. Whereas our most active preparations had a P.N. of about 1220, Theorell (1942*a*) regards P.N. 930 as the activity of the pure enzyme and ascribes his occasional higher values (1100) to catalytic impurities. We have confirmed the findings of previous workers (summarized by Ettori, 1949) namely, that the values of P.N. obtained in a series of estimations on the same sample under apparently

identical conditions can vary appreciably. In an attempt to make the P.N. estimation more reliable Sumner & Gjessing (1943) carried out the reaction in a small volume of buffer solution. This method was carefully examined by Ettori who also devised a manometric method based upon CO_2 production during the oxidation of pyrogallol but, according to him, neither method is free from the fluctuations of the normal test. In our experience, these fluctuations can be reduced to a large extent by the use of carefully purified pyrogallol (see Methods). Samples of pyrogallol which have not been so purified give rise to P.N. values which may be not only too high but also too low. Under these circumstances the discrepancies between the results of estimations of P.N. in different laboratories are in no way surprising.

One of the main objects of the present investigation was to determine the absolute absorption spectra of peroxidase and its derivatives both for the visible and the violet or Soret regions of the spectrum. It is important to note that in haemoproteins the most significant changes which are in one way or another associated with the iron atom are more clearly reflected in the general pattern of the absorption spectrum in the visible region than in the Soret band. In fact it was through the study of such changes in the visible region that the haematin nature of peroxidase was established and the formation of peroxidase-peroxide, in other words of an enzyme-substrate complex, was demonstrated for the first time (Keilin & Mann, 1937). It is also the existence of absorption bands in the visible region that makes possible the direct spectroscopic study of peroxidase in crude tissue extracts as well as in slices of horse-radish root, a property which will be valuable for the study of the physiological function of peroxidase.

Freezing and cooling to liquid air temperature of peroxidase and its derivatives in solutions or in slices of horse-radish greatly intensifies and sharpens the absorption bands. In this respect peroxidase behaves like other haematin compounds and many other pigments (Keilin & Hartree, 1949, 1950*a*). In slices cooled with liquid air even the band in the red of free peroxidase can easily be seen. The fact that similar cooling of alkaline peroxidase causes its absorption spectrum to change to that of the neutral form has been interpreted by us as indicating that the sixth co-ordination position around the haematin Fe is occupied by a molecule of water and not by a hydroxyl group as was postulated by Theorell & Paul (1944).

There is no evidence that the 'paraperoxidase' of Theorell (1940) occurs naturally in horse-radish roots, although in fact it may be formed in a solution of pure peroxidase which has been allowed to stand. It appears to be a reversible derivative of peroxidase in which the water molecule attached to the Fe atom is probably replaced by a group belonging to the protein.

Peroxidase forms with hydrogen peroxide one green (I) and two red (II and III) compounds. Since the green compound is the first to appear when peroxidase is treated with a small amount of H_2O_2 in absence of hydrogen donors, Theorell (1942*c*, 1947) suggested that this compound is the only catalytically active complex, i.e. the only one of the three which can react directly with hydrogen donors to yield oxidized donors and free peroxidase. However, this has not been confirmed by Chance (1949*c*) in the course of his study of the kinetics of reactions catalysed by peroxidase. Chance has been able to show that the rate of decomposition of the primary green compound (I) is proportional to the rate of formation and decomposition of the secondary red compound (II). The velocity of transition from I to II is greatly accelerated by the presence of a hydrogen donor and can even reach the velocity of formation of the primary compound. In the catalytic activity of peroxidase the complex II is thus the rate-determining Michaelis intermediate and during catalytic activity the enzyme is liberated not from the primary (I) but from the secondary (II) complex. This indicates that both complexes I and II are involved in the catalytic activity of the enzyme.

Comparison of peroxidase, catalase and methaemoglobin

In comparing these three haemoproteins the term methaemoglobin will be used in the widest sense to cover the oxidized or ferric forms of all haemoglobins. Catalase will also be used without distinction as to origin. On the other hand, the term peroxidase will be applied only to the enzyme obtained from horse-radish roots, since the knowledge so far available of other peroxidases, especially of their prosthetic groups, is far from complete.

We shall examine separately (*a*) the properties which are common to these haemoproteins and (*b*) the properties in which they differ.

(*a*) *Properties common to peroxidase, catalase and methaemoglobin*

(1) They are haemoprotein compounds having an identical prosthetic group, protohaematin, united to different proteins. (2) Their iron is in the trivalent state. (3) The colour and the general pattern of their absorption spectra are those of acid methaemoglobin (Fig. 5). (4) All three haemoproteins react reversibly with KCN, H_2S, NaF, NaN_3, NH_2OH, NO, H_2O_2 and ROOH (alkyl peroxides) forming spectroscopically well defined compounds, the formation of which requires 1 molecule of each of these substances per iron atom. (5) The cyanide and fluoride derivatives

of the three haemoproteins show very similar absorption spectra (Figs. 1, 6 and 7). (6) On denaturation or treatment with pyridine and $Na_2S_2O_4$ they give the same haemochromogen. They also yield the same haemin and the same porphyrin (protoporphyrin IX).

(b) *Differences in properties of peroxidase, catalase and methaemoglobin*

(1) Although the absorption spectra of these haemoproteins are of the same general pattern, there are certain interesting differences in the position and in the relative heights of their bands (Figs. 1–3, 5–7, also Table 2). The absorption spectrum of catalase in the visible region is represented in Fig. 5 by two curves obtained (i) from liver catalase, which contained a certain amount of verdo-haematin, and (ii) crystalline bacterial catalase, which is completely free from verdo-haematin (from the data of Herbert & Pinsent, 1948). As shown in Fig. 5, the band in the red (α) of catalase is much higher than those of methaemoglobin and peroxidase, the latter being the lowest of the three and lying nearer the red end of the spectrum. On the other hand, the relative heights of their Soret bands (γ) follow the

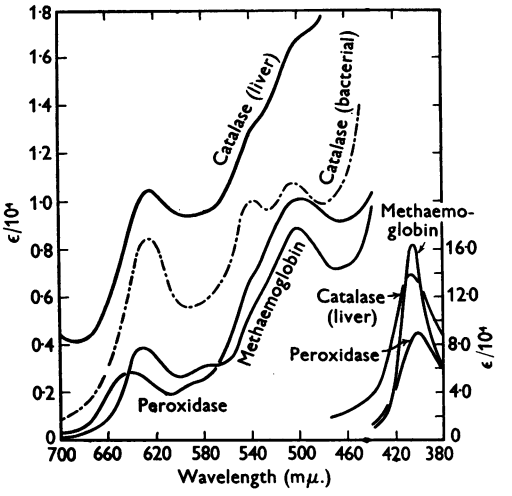

Fig. 5. Absolute absorption spectra of methaemoglobin, peroxidase and liver catalase (present investigation) together with the corresponding curve for bacterial catalase from the paper by Herbert & Pinsent (1948). For definition of units see Fig. 1.

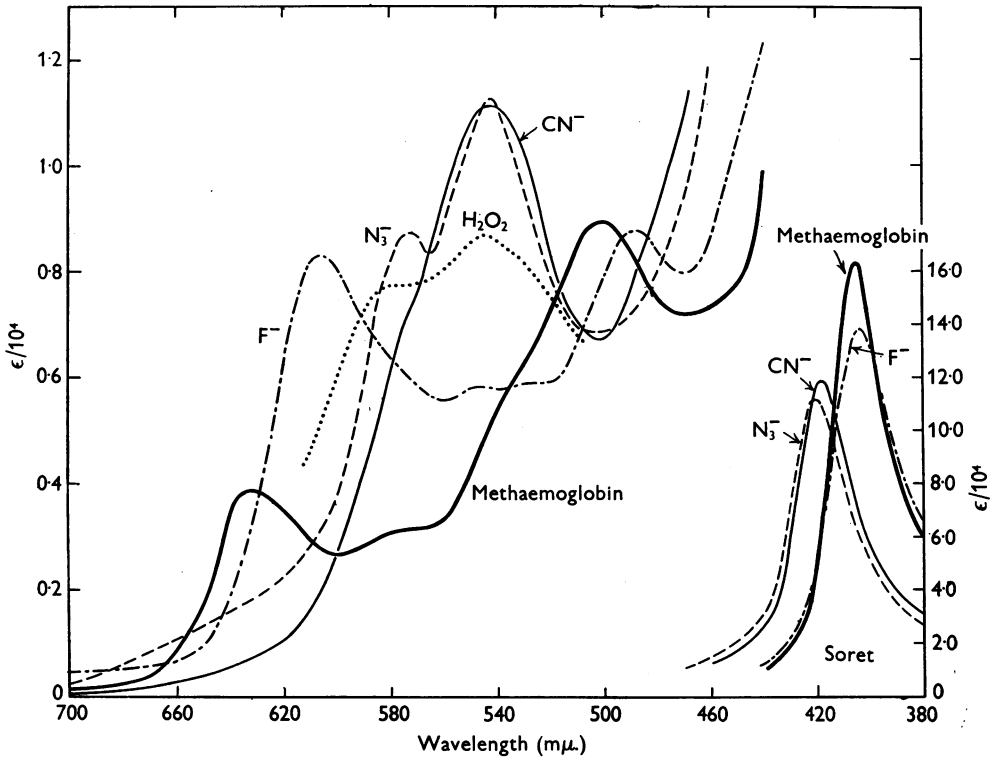

Fig. 6. Absolute absorption spectra at pH 6 of methaemoglobin (prepared from crystalline horse oxyhaemoglobin) and of its fluoride (F^-), cyanide (CN^-), azide (N_3^-) and H_2O_2 derivatives. For definition of units see Fig. 1.

order: Methb > catalase > peroxidase. The ratios of absorption coefficients $\epsilon_\gamma/\epsilon_\alpha$ for these three haemoproteins are: Methb 39, peroxidase 31 and catalase 9–14·2. So far no explanation can be offered for the much stronger absorption of light by catalase and its derivatives in the visible region compared with those of peroxidase and Methb.

(4) Methb (Fig. 6) and peroxidase (Fig. 1) on treatment with azide turn from brown to red and their absorption spectra are drastically changed. Catalase (Fig. 7), on the other hand, becomes somewhat more greenish and retains the same general unaffected by this reducing agent. In this respect catalase differs from all known haematin compounds.

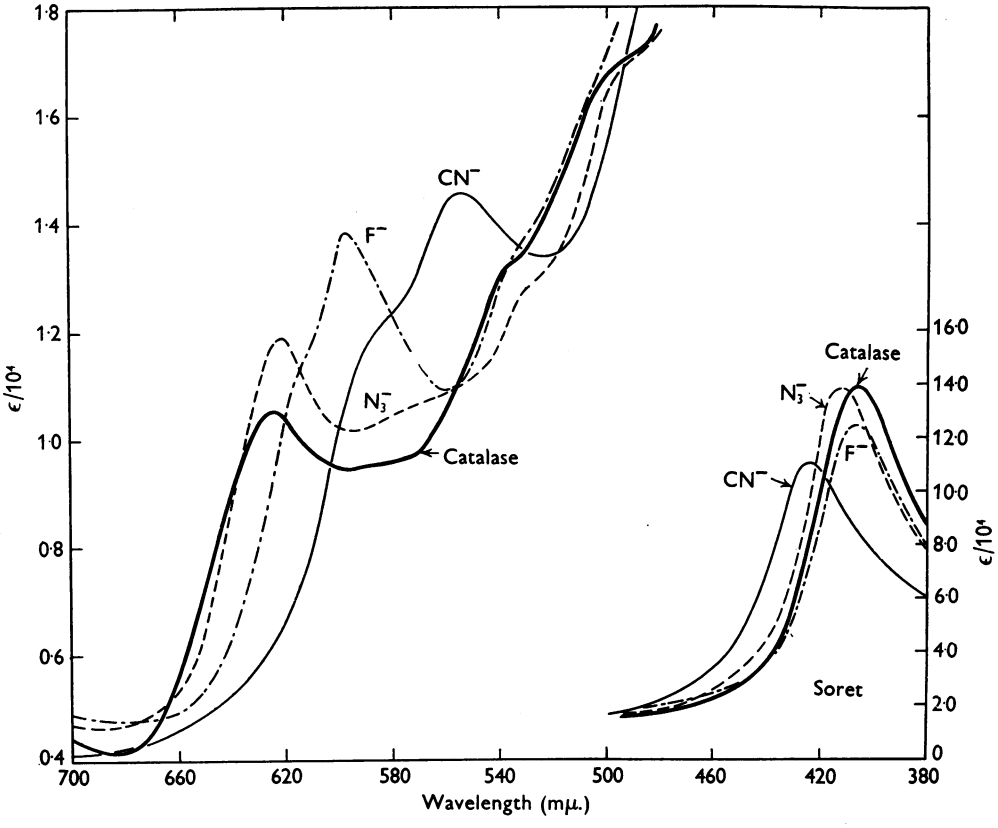

Fig. 7. Absolute absorption spectra at pH 6 of catalase (from horse liver) and of its fluoride (F^-), cyanide (CN^-) and azide (N_3^-) compounds. For definition of units see Fig. 1.

(2) On making a solution of Methb alkaline its colour changes from brown to red and its characteristic absorption spectrum is replaced by two wide bands at 545 and 575 mμ. and a narrow band at 600 mμ. Methb behaves as an indicator between pH 6·8 and 9·5 with pK = 8·1. Peroxidase, like Methb, shows changes in colour and absorption spectrum with changes of pH. At pH 11 a reddish alkaline peroxidase is obtained with bands at 574 and 545 mμ. (Fig. 2). On the other hand, the colour and absorption spectrum of catalase are only slightly affected by changes in pH.

(3) Methb and peroxidase (Fig. 2) are rapidly reduced by $Na_2S_2O_4$ from the ferric to the ferrous state as is shown by changes in their colours and absorption spectra, whereas catalase is completely spectral pattern, the only noteworthy change being that the band in the red becomes reinforced and shifted slightly towards the blue.

(5) Azide-catalase differs from azide-Methb and azide-peroxidase not only in its optical properties but also in its reaction with H_2O_2. Thus H_2O_2, when added to azide-Methb or azide-peroxidase, partially replaces the azide and forms the corresponding peroxide complexes in which the Fe remains trivalent. On the other hand, H_2O_2 reacts with azide-catalase to reduce its Fe to the divalent state, forming a compound which reacts both with oxygen and with CO. It was the study of this reaction which suggested that the catalatic decomposition of H_2O_2 by free catalase is also accompanied by valency changes of the iron (Keilin & Hartree, 1936*a*, 1945*a*).

Table 3. *Wavelengths (mμ.) of absorption bands in the visible region of complexes formed reversibly by peroxidase, catalase and Methb with* H_2O_2

		Complex II		Complex III	
	Complex I	α	β	α	β
Peroxidase	657	555*	527	583	546
Catalase	670 (?)	568	536	585	545
Methb	Not known	Not known		592*	545

* These bands are asymmetric and the figures given represent the wavelengths of maximum absorption and not the centres of the bands.

(6) The three haemoproteins form with H_2O_2 different complexes which, according to their absorption spectra in the visible region, can be classified as shown in Table 3. Thus peroxidase and catalase give three spectroscopically recognizable complexes of which peroxidase I and II and catalase I appear to be active in reactions which are so far known to be catalysed by these enzymes. Methb forms only one recognizable complex which corresponds to the type III given by peroxidase and catalase. On further reaction with H_2O_2 this Methb complex undergoes reduction to Hb and oxygenation to HbO_2, a reaction which is accompanied by catalatic decomposition of the H_2O_2.

(7) The most striking differences in the properties of these three haemoproteins are found in the magnitude of their catalytic activities. Whereas Methb shows only very slight peroxidatic and catalatic activities, peroxidase and catalase are very powerful catalysts.

For a long time the catalytic activities of peroxidase and catalase were considered to be fundamentally different, peroxidase utilizing H_2O_2 for the oxidation of various substances and catalase being capable only of decomposing H_2O_2 into oxygen and water. However, this difference lost all significance when catalase was shown to be capable of utilizing H_2O_2 for the peroxidatic oxidation of alcohols (Keilin & Hartree, 1936*b*, 1945*b*). The list of hydrogen donors for peroxidatic activity of catalase has since been extended (see p. 97) and now includes H_2O_2 itself (Chance, 1948, 1949*d*). The difference in the catalytic activities of peroxidase and catalase is therefore an expression of their specificity towards hydrogen donors undergoing peroxidatic oxidation.

Although the kinetics of the reactions of catalase and peroxidase with their substrate, and also with hydrogen donors and inhibitors, have been extensively studied the structures of the various enzyme-substrate intermediates remain obscure. Furthermore, in spite of the extensive investigations to which these enzymes have been subjected, work which has led to their complete purification and to a considerable knowledge of their constitution, very little is known of their role in cellular metabolism.

SUMMARY

1. A comparatively simple method is described for the isolation and purification of peroxidase from horse-radish roots.

2. The preparation contained 13·2% nitrogen and 1·61% haemin. The mean value of its activity in terms of Purpurogallin Number was 1220 and the molecular weight was about 40,000 (see Addendum, p. 105).

3. Absorption curves in the visible and Soret regions are given for peroxidase, for its alkaline and reduced forms, and for its compounds with carbon monoxide, cyanide, azide, fluoride and hydrogen peroxide.

4. The absorption bands in the visible region give a better indication than those in the Soret region of changes in the Fe linkages of peroxidase and its derivatives. These bands may be observed in crude enzyme preparations and even in slices of horse-radish root.

5. No evidence was found that 'paraperoxidase' exists as such in horse-radish roots, but it could be obtained as a transformation product of pure peroxidase.

6. Peroxidase catalyses the oxidation of dihydroxymaleic acid directly without the need for quinol or any other 'carrier'.

7. The effect of liquid air temperature on the absorption spectra of peroxidase and methaemoglobin suggests that the sixth co-ordination position around the iron atom in these haemoproteins, at a neutral or slightly acid pH, is a water molecule rather than a hydroxyl group.

8. Peroxidase gives rise successively on addition of hydrogen peroxide to three complexes which are easily recognized spectroscopically. Their absorption bands in the visible region are: I, approx. 657 mμ.; II, 555 and 527 mμ.; III, 583 and 546 mμ. Only I and II appear to take part in the normal catalytic activity of the enzyme. The band which appears at 670 mμ. as a result of prolonged treatment with hydrogen peroxide is probably that of a degradation product of peroxidase rather than a complex with hydrogen peroxide.

9. Catalase also forms with hydrogen peroxide three spectroscopically recognizable complexes with

the following bands: I, approx. 670 mμ.; II, 568 and 536 mμ.; III, 585 and 545 mμ. Complex I, which can only be detected by the rapid-flow technique, appears to be the only one that participates in the known reactions of this enzyme. Complex II can be obtained when hydrogen peroxide is supplied continuously by an oxidizing system; it can be converted to III by further reaction with hydrogen peroxide.

10. Methaemoglobin forms only one complex with hydrogen peroxide. The bands are at 592 and 545 mμ. and it thus corresponds to type III of peroxidase and catalase. The reaction of this complex with further hydrogen peroxide results in the reduction of methaemoglobin to haemoglobin, which can be detected as oxyhaemoglobin, and simultaneous liberation of oxygen from hydrogen peroxide.

11. Peroxidase resembles catalase and methaemoglobin in structure (all three being protein-protohaematin compounds), colour, general pattern of absorption spectra and formation of reversible and spectroscopically recognizable compounds with cyanide, sulphide, azide, fluoride, nitric oxide and peroxides.

12. Peroxidase and methaemoglobin, unlike catalase, can be reduced with sodium dithionite and show marked changes in colour and absorption spectrum with pH or on combining with azide. Azide-catalase, however, is spectroscopically very similar to free catalase and is reduced to a ferrous derivative by hydrogen peroxide.

13. There are no fundamental differences in the nature of the catalytic activities of peroxidase and catalase since the latter has been shown to catalyse peroxidatic oxidation of alcohols and other substances. However, the peroxidatic activity of catalase shows maximum efficiency only when hydrogen peroxide is continuously supplied by a system which reduces oxygen to hydrogen peroxide.

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ADDENDUM

Determination of Sedimentation and Diffusion Constants of Horse-Radish Peroxidase

By R. CECIL and A. G. OGSTON
Department of Biochemistry, University of Oxford

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Samples of peroxidase (fraction *H*), provided by Prof. D. Keilin and Dr E. F. Hartree were examined in a Svedberg oil-turbine ultracentrifuge and in the Gouy diffusiometer (Coulson, Cox, Ogston & Philpot, 1948). The buffer was NaCl, 0·2M; KH_2PO_4, 0·046M; Na_2HPO_4, 0·010M.

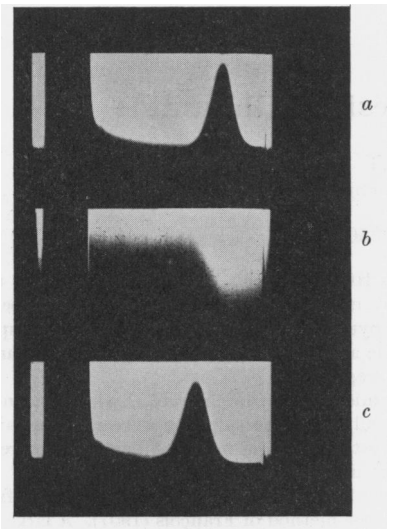

Fig. 1. Sedimentation diagrams: 40, 65 and 80 min. after reaching full speed; *a* and *c* by refraction, and *b* by absorption.

The sedimentation constant was first determined in a solution containing 7·7 mg./ml. at 1000 rev./sec.; observations were made alternately by the diagonal-schlieren method of Philpot (1938) at 600–640 mμ., and by absorption (Philpot, 1939) at 540–570 mμ. Fig. 1 shows refractometric and absorptiometric diagrams. In Fig. 2 the positions of the main boundary at successive times are shown plotted as $S(\Delta x)$ against $S(\Delta t')$ (Cecil & Ogston, 1948): the positions obtained from absorption lie closely on the same curve as those obtained refractometrically, showing that the absorbing material sediments exactly with the main refracting component. Of the total refracting material 92% forms the main sedimenting boundary (Cecil & Ogston, 1948), the remaining 8% being probably accounted for by a small amount of heterogeneous material. A second determination of the sedimentation constant, at a concentration of 3·4 mg./ml., was made for comparison with the diffusion constant determined at the same concentration.

Diffusion was observed by the Gouy method, using light of 622 mμ. (from a mercury lamp). At 6·9 mg./ml. the optical extinction of the solution was 0·37 and this proved to be too great for a really satisfactory record to be obtained: however, the result indicated that the material is nearly homo-

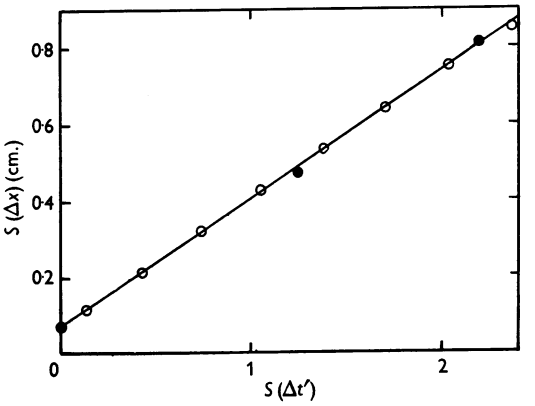

Fig. 2. Plot of $S(\Delta x)$ in cm. measured on the record against $S(\Delta t')$ in units of 10^{12}: ○, by refraction; ●, by absorption.

geneous with respect to diffusion. At 3·4 mg./ml. (extinction = 0·18) a satisfactory record was obtained. The theory of the interference of light in the Gouy method (Kegeles & Gosting, 1947) applies strictly only to the case where no absorption of light takes place: elementary theory (which may not be reliable) suggests that a moderate degree of absorption should not result in a large shift of the interference minima, and the results have been interpreted on this assumption.

The sedimentation and diffusion constants (corrected to their values for water at 20°) and other quantities are given in Table 1. The value of the partial specific volume was measured by Keilin & Hartree (1951) and agrees moderately well with the value 0·690 given by Theorell (1942).

Table 1. *Sedimentation and diffusion constants of peroxidase*

Concentration (mg./ml.)	S_{20} (corr.) ($\times 10^{13}$)	D_{20} (corr.) ($\times 10^{7}$)	$\bar{V}$	M	f/f_0	Proportion in main boundary
7·7	3·47	—	—	—	—	0·92
3·4	3·48	7·05	0·699	39,800	1·36	—

Theorell (1942) has reported sedimentation and diffusion measurements on peroxidase: our value for S_{20} (corr.) is about 10 % lower than his ($3{\cdot}85 \times 10^{-13}$) (cf. Cecil & Ogston, 1948), while our value for D_{20} (corr.) agrees fairly well with his value of $6{\cdot}84 \times 10^{-7}$. Our value for the molecular weight is lower than Theorell's (44,100), but is in agreement with that calculated by Keilin & Hartree (1951) from the haematin content; the chief uncertainty in our quantity probably lies in the value of $\bar{V}$.

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Studies on the Enzymic Synthesis of Glutamine

By W. H. ELLIOTT
Biochemical Laboratory, University of Cambridge

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Earlier reports described the discovery of an enzyme system in sheep brain which synthesizes glutamine in the presence of L-glutamic acid, adenosinetriphosphate (ATP) and ammonia (Elliott, 1948; Elliott & Gale, 1948). The present paper deals with the purification and detailed study of this enzyme system and the results of a distribution survey. A summary of this work was presented at the First International Congress of Biochemistry at Cambridge in August 1949 (Elliott, 1949). Speck (1947, 1949*a*, *b*) has independently reported a study of the glutamine-forming system present in pigeon liver.

MATERIALS

ATP was prepared as described by Needham (1942) except that in the final stages the method of Kerr (1941) was adopted to produce the monobarium salt. This was converted to the Na salt by dissolving it in N-HCl and adding a slight excess of Na_2SO_4. After centrifuging, the supernatant was neutralized to pH 6·8 and stored at −10°. The solution used contained 0·1 mg. inorganic P/ml. and 4·3 mg. of P hydrolysed by boiling N-HCl in 10 min. (acid-labile P). The solution was analysed enzymically by the method of Bailey (1949). The percentages of the acid-labile P hydrolysed by myosin, by myosin + myokinase, and by inorganic pyrophosphatase were 45·6, 91·2 and 2·6 respectively.

Adenosinediphosphate (ADP) was prepared from ATP by the method of Bailey (1942). The Ba salt was treated in the same way as that for ATP. The solution used contained 3·16 mg. of total P/ml., of which 50 % was hydrolysed by boiling N-HCl in 10 min. The percentages of the total P split by myosin alone, by myosin + myokinase, and by inorganic pyrophosphatase were 1·0, 47 and 0·02 respectively.

Adenylic acid (AA) was supplied by the Armour Laboratories, Chicago.

L-Glutamic acid hydrochloride, hydroxylamine hydrochloride and hydrazine sulphate were commercial samples twice recrystallized from water. All solutions were neutralized before use.

Methylamine hydrochloride was prepared free from NH_4Cl by the method of François (1907). A large excess of yellow HgO was used to make sure that all traces of NH_3 were removed.

DL-Methionine oxide was prepared by the method of Toennies & Kolb (1939).

D-Glutamic acid, *N*-carbobenzyloxy-L-glutamic acid, L-*iso*glutamine (L-glutamic acid α-amide), and L-glutamine were kindly given by Dr A. C. Chibnall.

Phosphate buffers were mixtures of Na_2HPO_4 and KH_2PO_4.

Glyoxaline buffers were prepared by adjusting the pH of solutions of the base with HCl.

METHODS

Incubations were usually performed in open tubes at 30° and samples taken as necessary for estimations.

Chemical determinations

Hydroxamic acid was estimated by the method of Lipmann & Tuttle (1945) except that determinations were done on 4·5 ml. samples as described previously (Elliott & Gale, 1948).